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Quaranfil, Johnston Atoll, and Lake Chad Viruses Are Novel Members of the Family *Orthomyxoviridae*▽

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Arboviral infections are an important cause of emerging infections due to the movements of humans, animals, and hematophagous arthropods. Quaranfil virus (QRFV) is an unclassified arbovirus originally isolated from children with mild febrile illness in Quaranfil, Egypt, in 1953. It has subsequently been isolated in multiple geographic areas from ticks and birds. We used high-throughput sequencing to classify QRFV as a novel orthomyxovirus. The genome of this virus is comprised of multiple RNA segments; five were completely sequenced. Proteins with limited amino acid similarity to conserved domains in polymerase (PA, PB1, and PB2) and hemagglutinin (HA) genes from known orthomyxoviruses were predicted to be present in four of the segments. The fifth sequenced segment shared no detectable similarity to any protein and is of uncertain function. The end-terminal sequences of QRFV are conserved between segments and are different from those of the known orthomyxovirus genera. QRFV is known to cross-react serologically with two other unclassified viruses, Johnston Atoll virus (JAV) and Lake Chad virus (LKCV). The complete open reading frames of PB1 and HA were sequenced for JAV, while a fragment of PB1 of LKCV was identified by mass sequencing. QRFV and JAV PB1 and HA shared 80% and 70% amino acid identity to each other, respectively; the LKCV PB1 fragment shared 83% amino acid identity with the corresponding region of QRFV PB1. Based on phylogenetic analyses, virion ultrastructural features, and the unique end-terminal sequences identified, we propose that QRFV, JAV, and LKCV comprise a novel genus of the family *Orthomyxoviridae*.

Arboviral infections are an important and emerging cause of human illness. Recent epidemics of West Nile, chikungunya, dengue, and yellow fever illustrate the importance of understanding the basic virology of arboviruses. Quaranfil virus (QRFV) is a heretofore-unclassified arbovirus isolated in 1953 from ticks (*Argas* [*Persicargus*] *arboreus*) collected near Cairo, Egypt, and subsequently passaged in mice and Vero cells. This tick-derived isolate was determined by serologic methods to be related to a virus previously cultured from the blood of two children with mild febrile illnesses in Quaranfil, Egypt (32). Human serologic studies performed in the 1960s in Egypt revealed that approximately 8% of the local population had neutralizing antibodies to this virus, demonstrating that human infection occurs and raising the question of whether QRFV might represent an unrecognized cause of viral illness in humans (32). The extent to which this virus may cause clinical illness in people is currently unknown; however, multiple strains of QRFV have been isolated from ticks and seabirds in Egypt, South Africa, Afghanistan, Nigeria, Kuwait, Iraq, Yemen, and Iran (1, 12, 20, 29, 30). QRFV is lethal after intracerebral (i.c.) inoculation of newborn mice (32). One study reported that experimental QRFV infection of laboratory mice causes a lethal respiratory disease and meningoencephalitis (4).

Johnston Atoll virus (JAV) was originally isolated from ticks (*Ornithodoros capensis*) collected in 1964 from a Noddy Tern (*Anous stolidus*) nest, Sand Island, Johnston Atoll in the central Pacific (11). Multiple strains have subsequently been isolated from eastern Australia, New Zealand, and Hawaii (3). No human disease has been associated with JAV, but it is lethal to newborn and weanling mice after i.c. injection and to 1- to 2-day-old chicks after subcutaneous inoculation. (11). Lake Chad virus (LKCV), strain Ib An 38918, was isolated from a masked weaver bird, *Ploceus vitellinus*, collected at Lake Chad, Nigeria, in 1969. LKCV is lethal to newborn mice after i.c. inoculation, and it was shown to be antigenically related to QRFV (R. E. Shope, personnel communication).

To date, conventional approaches to characterize QRFV, JAV, and LKCV have not resulted in a definitive classification of these viruses. QRFV and JAV are enveloped RNA viruses, and electron microscopic and serologic studies tentatively suggested a classification in the arenavirus family based on morphological and morphogenetic features of the viruses (38). In this study, we utilized high-throughput sequencing to identify genomic sequences from QRFV, JAV, and LKCV. Based on analysis of the complete sequences of five of these segments from QRFV and partial sequences from JAV and LKCV, ultrastructural analysis of infected cell cultures, and serologic testing, we propose that these viruses define a novel genus in the family *Orthomyxoviridae*.

MATERIALS AND METHODS

Virus strains and propagation. QRFV (strain EG T 377, originally isolated from a tick collected in Afghanistan in 1968) was propagated in Vero cells and 3T12 fibroblasts. JAV (strain LBJ) and LKCV (strain Ib An 38918) were prop-

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agated in Vero cells. All viruses were obtained from the World Reference Center for Arboviruses and Emerging Viruses at the University of Texas Medical Branch (UTMB), Galveston, TX.

Serologic testing. Complement fixation (CF) tests were done by a microtechnique (5, 10, 13, 37), using 2 full units of complement. Titers were recorded as the highest dilutions giving 3+ or 4+ fixation of complement on a scale of 0 (complete hemolysis) to 4+ (no hemolysis). For CF tests, titers of ≥1:8 were considered positive. Hemagglutination inhibition (HI) testing was done in microtiter plates as described previously (5, 10, 13, 37). HI tests were performed with 4 hemagglutination units of virus at the optimal pH (5.75) against serial twofold antiserum dilutions starting at 1:20. HI titers of ≥1:20 were considered positive.

Cell culture. Vero cells, 3T12 fibroblasts, HeLa cells, MDCK cells, and C6/36 cells were all obtained initially from the ATCC. Murine embryonic fibroblasts were prepared as previously described (27). All cells were propagated in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (HyClone), 1% penicillin-streptomycin (Cellgro), 200 mM L-glutamine (Gibco), and 1% HEPES (Cellgro) (D10 medium). All cultures were maintained at 37°C with an atmosphere of 5% CO_2, except for C6/36 cells, which were maintained at 30°C.

Animal infection and antisera. QRFV-specific antibodies were generated at Washington University or UTMB in accordance with all federal and university policies. QRFV-specific antiserum used for cryo-immunoelectron microscopy was generated in C57BL/6 mice at Washington University. In order to avoid cross-reactivity with cellular antigens in the infected 3T12 cells, we repeatedly infected mice with supernatant derived from QRFV-infected Vero cells. Mice were simultaneously infected by intraperitoneal and intranasal routes with 100 μl intraperitoneally and 40 μl intranasally (total of ~1,000 50% tissue culture infective doses [$TCID_{50}$]) of supernatant derived from QRFV-infected Vero cells, and then boosted by infection every month for 3 months with 100 μl supernatant intraperitoneally. Antiserum was harvested at 4 months after the initial infection. This antiserum is capable of neutralizing 1,000 $TCID_{50}$ of QRFV at a dilution of 1:640 and maintains specific signal in enzyme-linked immunosorbent assays against infected 3T12 lysates at a dilution of 1:1,000.

Hyperimmune ascitic fluid used for serologic testing was prepared in Institute for Cancer Research (ICR) mice obtained from Harlan Sprague-Dawley (Indianapolis, IN) at UTMB as described previously (37). The immunizing antigens for antibody production were prepared from brains of newborn mice inoculated intracerebrally with JAV, LKCV, and QRFV.

Viral growth and titer. The virus titer was determined according to the $TCID_{50}$ calculated according to the method of Reed and Muench (28). Six 100-μl replicates of 10-fold dilutions of samples were plated in 96-well plates. 3T12 fibroblasts were added at a concentration of 3,000 cells/well in 100 μl. Wells were scored for cytopathic effect (CPE) at 5 days postinfection. Multistep growth curves for QRFV were performed by infecting 50,000 cells per well in 1 ml plated in 24-well plates. Cells were infected with 500 $TCID_{50}$ QRFV at 37°C for 2 h, and then virus was removed and 1 ml D10 medium replaced per well. In order to perform a time course experiment, infected plates were frozen at −80°C at various time points postinfection. All plates in a given time course were thawed and the virus titer determined within the same experiment. Single-step growth curves for QRFV were performed in 3T12 cells. Cells were plated as described above but were infected at a multiplicity of infection of 5.

Transmission electron microscopy. For ultrastructural analysis, 3T12 cells were infected with 0.01 $TCID_{50}$ per cell and harvested at 5 days postinfection. They were fixed in 2% paraformaldehyde–2.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM phosphate buffer, pH 7.2, for 1 h at room temperature. Samples were washed in phosphate buffer and postfixed in 1% osmium tetroxide (Polysciences Inc., Warrington, PA) for 1 h. Samples were then rinsed extensively in distilled water prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 h. Following several rinses in distilled water, samples were dehydrated in a graded series of ethanol solutions and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA).

Cryo-immunoelectron microscopy. For immunolocalization of antiviral antibody at the ultrastructural level, cells were infected with 0.01 $TCID_{50}$ per cell, harvested at 5 days postinfection, and fixed in 4% paraformaldehyde–0.05% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM PIPES–0.5 mM $MgCl_2$, pH 7.2, for 1 h at 4°C. Samples were then embedded in 10% gelatin and infiltrated overnight with 2.3 M sucrose–20% polyvinyl pyrrolidone in PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]–$MgCl_2$ at 4°C. Samples were trimmed, frozen in liquid nitrogen, and sectioned with a Leica Ultracut UCT cryo-ultramicrotome (Leica Microsystems Inc., Bannockburn, IL). Sections of 50 nm were blocked with 5% fetal bovine serum–5% normal goat serum for 30 min and subsequently incubated with a 1:500 dilution of mouse anti-QRFV antiserum for 1 h at room temperature. Sections were then washed in block buffer and probed with 18-nm colloidal gold-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove PA) for 1 h at room temperature. Sections were washed in PIPES buffer, rinsed with water, and stained with 0.3% uranyl acetate–2% polyvinyl alcohol. Samples were viewed with a JEOL 1200EX transmission electron microscope (JEOL USA Inc., Peabody, MA). All labeling experiments were conducted in parallel with controls omitting the primary antibody. These controls were consistently negative at the concentration of colloidal gold-conjugated secondary antibodies used in these studies.

RNA extraction. Total RNA from infected cells was used for shotgun sequencing by the Sanger method and for reverse transcription-PCR (RT-PCR) and rapid amplification of cDNA ends (RACE). Briefly, RNA was extracted from Vero or 3T12 cells that were mock infected or infected with QRFV or JAV using Trizol (Invitrogen, Carlsbad, CA). The sample was mixed with chloroform (1/5 volume) and centrifuged at 12,000 × *g* for 15 min at 4°C. The aqueous phase was transferred, mixed with an equal volume of isopropanol, and centrifuged at 12,000 × *g* for 15 min at 4°C. The RNA pellet was washed with 70% ethanol and then dissolved in nuclease-free water. Viral RNA was prepared for 454 sequencing as follows. 3T12 cells were infected with 0.01 $TCID_{50}$/cell and harvested when 75% of the cells demonstrated CPE (~5 to 6 days). The cells and supernatant were harvested and spun at 1,500 × *g* for 20 min at 25°C. The supernatant was then spun at 13,600 × *g* for 2 h at 4°C. The virus-containing pellet was then resuspended in 2 ml of DNase I buffer and treated with 1.4 μg/ml DNase I (Gibco) for 30 min at 37°C. RNA was then prepared using the QIAmp MinElute virus spin kit (Qiagen).

RT, amplification, and sequencing. Random amplification of the extracted RNA was performed as described previously (34). Briefly, RNA was reverse transcribed with primer A (5′-GTTTCCCAGTCACGATAN$_9$), and second-strand DNA synthesis was carried out with Sequenase (U.S. Biochemical, Cleveland, OH). This material was used as the template for 40 cycles of PCR with primer B (5′-GTTTCCCAGTCACGATA) using the following program: 30 s at 94°C, 30 s at 40°C, 30 s at 50°C, and 60 s at 72°C. The amplified material was either cloned into pCR4-TOPO and sequenced using Sanger chemistry on an ABI 3730 × 1 sequencer (Applied Biosystems) or pyrosequenced using a GS FLX platform (454 Life Sciences; Roche). Sequences were then trimmed to remove vector and/or primer B sequences. CD-HIT was used to remove redundant sequences, and repetitive sequence was masked using RepeatMasker (www.repeatmasker.org) to generate a high-quality data set. This data set was then analyzed using BLASTn and tBLASTx (2). Contigs were then assembled using Newbler (454 Life Sciences).

Viral segment sequencing. Assembled contigs of PB1 and hemagglutinin (HA) genes from Sanger sequencing and pyrosequencing were confirmed by RT-PCR followed by sequencing. Gaps between contigs were closed by designing PCR primers from the existing contigs. PCR amplicons were cloned into pCR4-TOPO and sequenced. The ends of each genomic segment were obtained by performing 5′ RACE using the 5′ RACE system (Invitrogen). 5′ RACE was performed using primers directed at amplifying either the 5′ end of the genome or the antigenome present as viral mRNA derived from infected cells. Additional segments were obtained by RT-PCR using the primer GTCCCAGTCACGATCAGCAAATC ACAAWYCTCT, which contains the terminal conserved sequence identified by 5′ RACE analysis as well as additional 5′ sequence to provide a higher melting temperature for PCR. RT was performed using this gene-specific primer with either SuperScript II at 42°C for 50 min or Superscript III at 50°C for 50 min (Invitrogen), with similar results. PCR with the same primer was performed for 35 cycles using the following program: 30 s at 94°C, 30 s at 55°C, and 120 s at 72°C. Either the entire reaction product or individual gel-purified bands were cloned into pCR4-TOPO and sequenced.

Phylogenetic analysis. The amino acid sequences for the largest predicted open reading frames (ORFs) of the QRFV and JAV segments were aligned with sequences from the NCBI reference genome sequences for influenza A virus (PB1, NC_007358, NC_04911, NC_007375, NC_007372, and NC_002021; PB2, YP_308849.1, YP_308855.1, YP_308664.1, NP_859039.1, and NP_040987.1; PA, YP_308852.1, YP_308846.1, YP_308666.1, NP_859041.1, and NP_040986.1; HA, NC_007362 [H5], NC_004908 [H9], NC_007374 [H2], NC_007366 [H3], and NC_002017 [H1]), influenza B virus (PB1, NC_002204; PB2, NP_056658.1; PA, NP_056659.1; and HA, NC_002207), influenza C virus (PB1, NC_006308; PB2, YP_089652.1; PA, YP_089654.1; and HA, NC_006310), Thogoto virus (PB1, NC_006495; PB2, YP_145810.1; PA, YP_145795.1; and HA, NC_006506), and infectious salmon anemia virus (PB1, NC_006503; PB2, YP_145807.1; PA, YP_145802.1; and HA, NC_006501). Since no reference genome has been de-

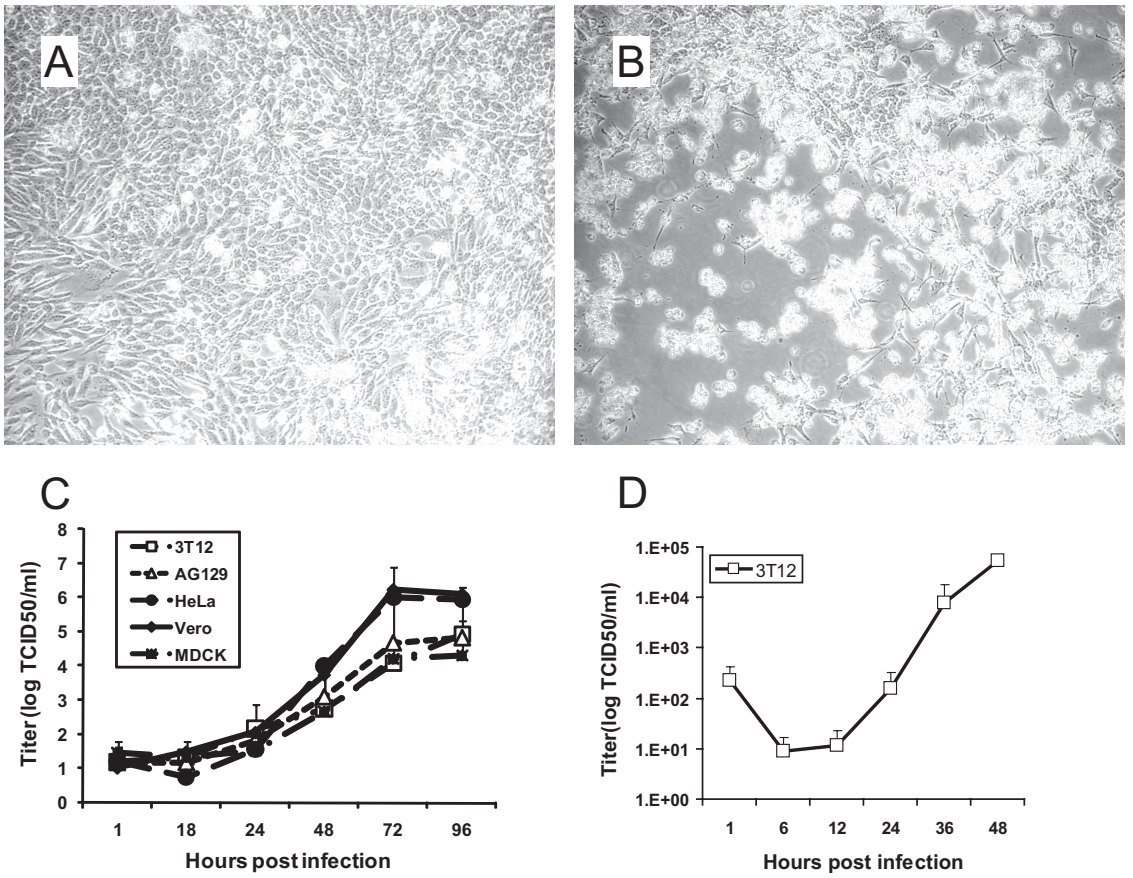

FIG. 1. Growth and CPE of QRFV. (A) Uninfected 3T12 fibroblasts. (B) QRFV-infected fibroblasts at 5 days postinfection with multiplicity of infection of 0.01. (C) Multistep growth of QRFV in several cell types. The data shown are pooled from three independent experiments. Error bars indicate standard deviations. (D) Single-step growth for QRFV in 3T12 cells. The data shown are from one representative of two independent experiments.

termined for Dhori virus, its sequence was obtained from the GenBank protein database (PB1, AAA42968; HA, AAA47907 [PB2 and PA sequences have not been submitted]). Alignments were performed using ClustalX V1.83 (9) with default parameters. Phylogenetic trees were constructed using neighbor-joining methods (9), maximum-parsimony methods using PAUP 4.0 (31), or maximum likelihood with PhyML (15). Default settings were used for each method, and 1,000 bootstrap repetitions were performed. Trees with the same topology were generated using all three methods.

Nucleotide sequence accession numbers. Sequences of QRFV (PB1, FJ861695; HA, FJ861694; PA, GQ499303; PB2, GQ499302; segment 5, GQ499304), JAV (PB1, FJ861697; HA, FJ861696), and LKCV (PB1, FJ861698) have been deposited in GenBank.

RESULTS

Growth and CPE of QRFV. QRFV has previously been passaged through Vero cells and by transfer through neonatal mice (4, 32). We passaged it in multiple cell types, including murine (3T12 or primary embryonic fibroblasts), canine (MDCK), human (HeLa), primate (Vero), and insect (C6/36) cell lines. CPE was not observed in infected MDCK, HeLa, or C6/36 cells. Minimal CPE was seen in Vero cells. In contrast, distinctive, plaque-like CPE was seen in the murine fibroblast lines, initially at 7 days postinfection and then at 3 days postinfection on passage (Fig. 1B). In an effort to more completely understand the growth features of QRFV, we developed a $TCID_{50}$ assay, taking advantage of the CPE of QRFV in 3T12 cells. Multistep growth curves were performed with the cell lines described above (Fig. 1C). Similar kinetics of growth was seen in all lines tested, except that no growth was detected in C6/36 cells. We consistently observed a 1- to 1.5-log-lower titer in the murine cell lines than in HeLa or Vero cells. This ability to grow in multiple mammalian lines, but not in the mosquito line C6/36, is consistent with what has been described for tick-borne orthomyxoviruses, such as Thogoto virus and Araguari virus (13, 38). Single-step growth curves in 3T12 cells yielded a replication cycle of approximately 24 to 36 h, slower than that of the influenza viruses but similar to that of tick-borne orthomyxoviruses (7, 13, 38) (Fig. 1D).

Electron microscopic studies of QRFV-infected 3T12 cells. Electron microscopy was performed on QRFV-infected 3T12 cells at 5 days postinfection, at which time ~75% of the cells are lysed by CPE. Electron microscopy of conventional ultrathin sections of infected, but not uninfected, 3T12 cells revealed pleomorphic virus particles which were approximately 100 nm in size and budding from the cell, similar to those observed for other orthomyxoviruses (21) (Fig. 2A and B). Capsid-like particles were seen in the cytoplasm of infected cells (data not shown). QRFV-specific antiserum was used to

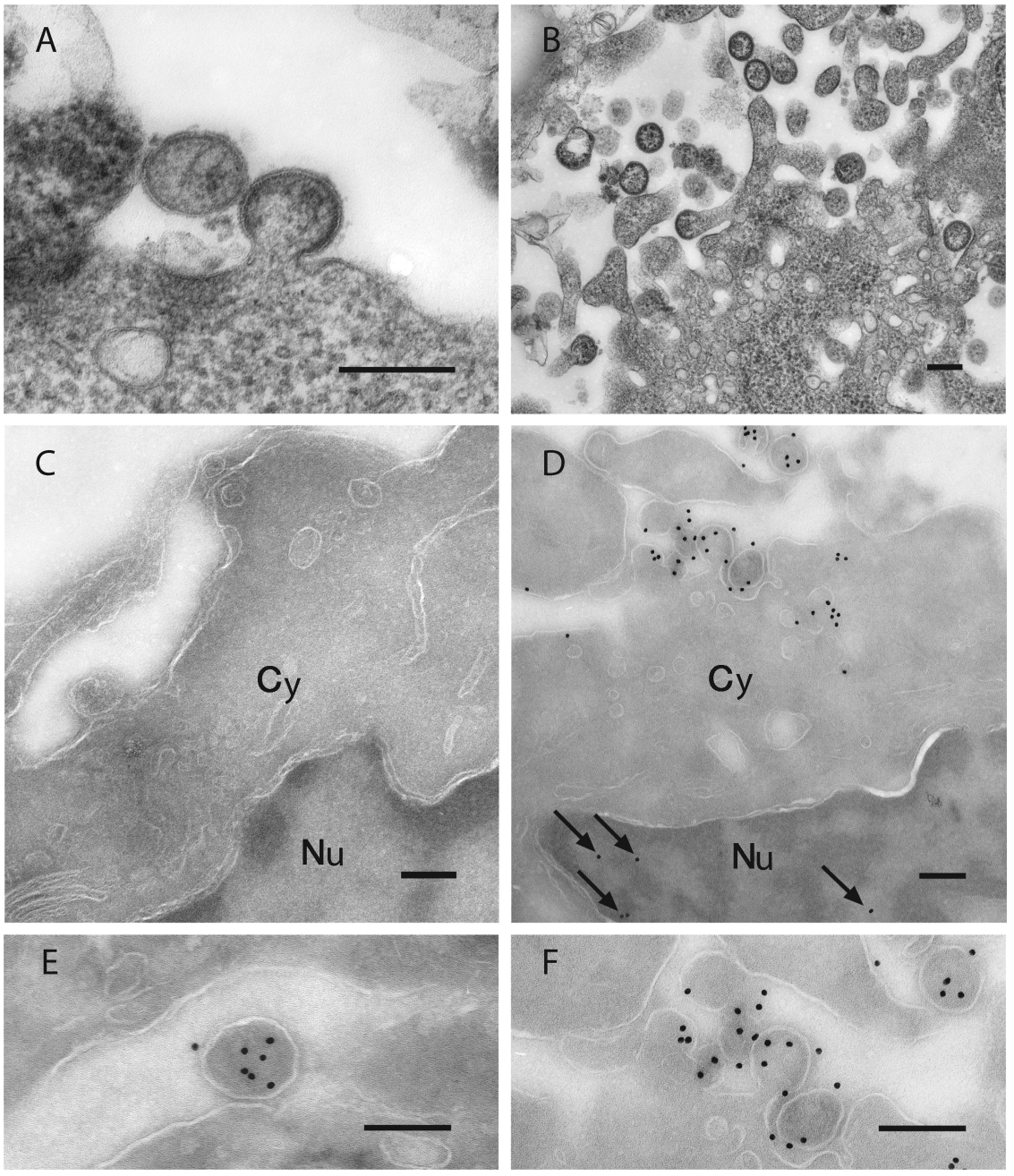

FIG. 2. Electron microscopy of QRFV. (A and B) Resin-embedded QRFV-infected 3T12 fibroblasts at 5 days postinfection. (C) Cryo-immunoelectron microscopy of uninfected 3T12 fibroblasts labeled with anti-QRFV antibody. (D to F) Cryo-immunoelectron microscopy of QRFV-infected 3T12 fibroblasts labeled with anti-QRFV antibody. Cy, cytoplasm; Nu, nucleus. Bars, 200 nm.

stain QRFV-infected 3T12 fibroblasts using immunogold labeling on ultrathin cryosections, demonstrating that the orthomyxovirus-like particles seen by electron microscopy were QRFV (Fig. 2D to F). Uninfected cells had no virus-like particles and showed no evidence of staining with QRFV-specific antiserum (Fig. 2C). Evidence of immunogold-stained virus-like particles in both the nuclei and cytoplasm of infected cells was detected, consistent with what is known about the viral life cycle of orthomyxoviruses (Fig. 2D).

Sequencing of QRFV HA and PB1. Because previous efforts to characterize QRFV using electron microscopy and serology had not yielded a definitive classification, we used a mass-sequencing approach to characterize these viruses. Sequence from the QRFV genome was obtained by Sanger sequencing and pyrosequencing of reverse-transcribed and randomly amplified RNA from infected cell cultures. No similarity to any known virus was detected at the nucleotide level using BLASTn (2). However, by translating the assembled se-

TABLE 1. Nucleotide and amino acid contents of virus segments

Virus	nt/aa					
	PB1	PB2	PA	HA	NP	M
Influenza A virus	2,341/757	2,341/759	2,233/716	1,760/566	1,557/498	1,027/252
Influenza B virus	2,368/752	2,313/770	2,204/726	1,882/584	1,841/560	1,191/248
Influenza C virus	2,265/754	2,325/774	2,130/709	1,968/655	1,807/565	1,125/242
Thogoto virus	2,212/710	2,375/769	1,927/622	1,574/512	1,418/454	955/266
Infectious salmon anemia virus	2,185/708	2,169/722	1,787/578	1,323/394	2,046/616	—[a]
QRFV (putative)	2,386/777	2,421/733	2,403/688	1,616/511		900/266[b]

[a] The matrix protein for infectious salmon anemia virus was originally thought to be encoded by segment 7 (966 nt). However, antibodies generated against the protein predicted by the largest ORF do not react with purified virus, suggesting that segment 7 encodes nonstructural proteins (25).
[b] Classification of segment 5 is based on size similarity, not structural similarity, to orthomyxovirus matrix proteins.

quences, we identified three sequences of 590, 524, and 225 bp which had regions with 30 to 45% amino acid identity to the polymerase gene, PB1, from influenza B virus. In addition, one assembled sequence of 1,392 bp had two subsequences which were predicted to encode a protein with 30 to 40% amino acid identity to viral glycoproteins from baculovirus. This protein sequence also shared limited identity to the HA glycoprotein of the thogotoviruses. The QRFV sequences identified were specific to virus-infected cultures and could not be found in uninfected 3T12 cells. Elaboration of these QRFV sequences by RT-PCR and RACE yielded the complete sequences of two segments of the QRFV genome. One segment (PB1) was 2,386 nucleotides (nt) long, and contained one predicted ORF encoding 777 amino acids (aa). The other segment (HA) was 1,616 nt long and contained one predicted ORF encoding 511 aa. These lengths are comparable to those seen in the corresponding genomic segments from orthomyxoviruses (Table 1). PB1 is the most conserved of the orthomyxovirus genes, and is therefore a robust marker for the phylogenetic relationships among the different genera of the *Orthomyxoviridae* (22). Phylogenetic analysis of PB1 demonstrated that QRFV is highly divergent from viruses in the other genera of the *Orthomyxoviridae* (Fig. 3A). Phylogenetic analysis of HA gave a similar pattern of divergence between QRFV and the other orthomyxoviruses, although HA from QRFV is more closely related to the thogotoviruses than to the influenza viruses (Fig. 3B).

QRFV segment terminal sequences. One of the defining features of the *Orthomyxoviridae* family is the presence of highly conserved sequences at the ends of each segment (Table 2). These sequences show partial inverted complementarity and are thought to be critically important to the binding and activation of the viral polymerase proteins (6, 16, 33). To obtain the terminal sequences, we performed 5′ RACE analysis targeting both the sense and antisense strands, presumed to be the viral mRNA and the viral genomic RNA. RACE analysis of the 5′ ends of the viral genomic RNAs of PB1 and HA segments of QRFV showed the sequence 5′-AGCAAUCACAA-(A/U)(U/C)CUCUUUUUU, followed by segment-specific sequence. Consistent with this, 3′ RACE of this region of QRFV HA revealed a polyadenylation tract which appeared to start from this polyuridine tract, consistent with the viral genomic RNA being antisense. 5′ RACE of the viral mRNA revealed the following pattern: 9 to 11 nt which were not conserved among different RACE products (Table 3), then the sequence 5′-AGCAAUCACA(A/U)(U/C)CUCUUU, followed by segment-specific sequence. Thus, the conserved 3′ sequence at the end of the viral genome has the sequence 3′UCGUUAGUG U(U/A)(A/G)GAGAAA, which has partial inverted complementarity to the 5′ end of the viral genome. The presence of 9 to 11 nt of heterogeneous sequence at the 5′ end of the mRNA

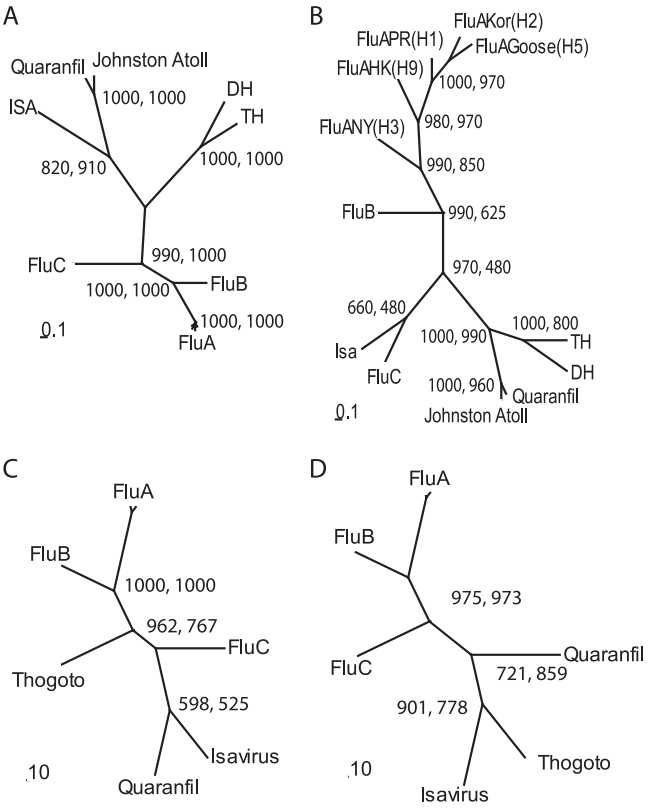

FIG. 3. Phylogenetic analysis of the conserved viral proteins. Phylogenetic trees were constructed with the sequences for homologous proteins. (A) PB1; (B) HA; (C) PA; (D) PB2. Trees were generated using the maximum-parsimony and maximum-likelihood methods with 1,000 bootstrap replicates. The most parsimonious trees are shown. The numbers at the branches show bootstrap values for both methods (maximum parsimony, maximum likelihood). FluC, influenza C virus (C/Ann Arbor/1/50); FluB, influenza B virus (B/Lee/40); Th, Thogoto virus (SiAr 126); Dh, Dhori virus (Indian/1313/61); Isa, infectious salmon anemia virus; Quaranfil, QRFV (EG T 377); Johnston Atoll, JAV (South Pacific strain). For the polymerase sequences, the five reference strains of influenza A virus (A/Puerto Rico/8/34 [H1N1], A/New York/392/2004 [H3N2], A/Hong Kong/1073/99 [H9N2], A/Goose/Guangdong/1/96 [H5N1], and A/Korea/426/68 [H2N2]) grouped closely, and for clarity, only FluA is indicated.

TABLE 2. Sequences of segment termini

Virus	Sequence	
	5′	3′
Influenza A virus	AGUAGAAACAA	UCGYUUUCGUCC
Influenza B virus	AGUAGWAACAA	UCGUCUUCGCKU
Influenza C virus	AGCAGUAGCAA	UCGUYUUCGUCC
Thogoto virus	AGAGAAAUCAA	UCGUUUUUGUCC
QRFV	AGCAAUCACAA	UCGUUAGUGUWR

is consistent with what is seen in the influenza viruses (14) and in contrast to the case for Thogoto virus (35, 36). Of note, the conserved terminal sequences seen in QRFV are not the same as conserved sequence motifs seen at the ends of sequences of any other genus of orthomyxoviruses (Table 2), suggesting that QRFV is not a member of any of the known genera of orthomyxoviruses.

Sequencing of additional segments of QRFV. To identify and obtain the sequences of additional segments of QRFV, we performed RT-PCR using a primer which contained the conserved sequence present at the end of the putative HA and PB1 segments. We consistently obtained four distinct bands of approximately 2.4 kb, 1.6 kb, 900 bp, and 450 bp which were not amplified in RNA from uninfected 3T12 cells. Cloning and sequencing of either these bands or the entire RT-PCR product, followed by BLAST analysis as described above, resulted in the identification of three additional segments, consistent with QRFV having at least five segments in its genome. The largest of the novel viral segments identified was 2,421 nt long and predicted a single ORF encoding of 733 aa. Analysis using tBLASTx against either the entire nucleotide database or the *Orthomyxoviridae* database (taxid:11308), did not result in the identification of any sequence with significant homology using a cutoff E of <1e−3. Translation of the predicted ORF and analysis using the Conserved Domain Database (CDD) within the BLASTp suite (23, 24) did reveal the presence of a domain consistent with the Flu_PB2 superfamily (pfam00604), which hit with an E value of 2e−07. The second segment was 2,403 nt long and predicted one ORF encoding 688 aa and a second smaller ORF downstream of this encoding 76 aa. Analysis using tBLASTx against either the entire nucleotide database or the *Orthomyxoviridae* database (taxid:11308) did not result in the identification of any sequence with significant homology using a cutoff E of <1e−3. Translation of the larger ORF and analysis using CDD identified a putative conserved domain hit for the Flu_PA superfamily (pfam00603), with an E value of 0.003. Phylogenetic analysis of these two predicted proteins compared to PA and PB2 from the reference sequences from other orthomyxoviruses confirms the divergence of QRFV from the known genera (Fig. 3C and D). A fifth segment was also identified, with a length of 900 nt, predicting a single ORF encoding 266 aa. Analysis using tBLASTx identified no significant homologs in either the nucleotide or orthomyxovirus database. No conserved domains were detected using CDD. No significant features were identified using Prosite (18, 19).

TABLE 3. 5′ RACE mRNA

mRNA	Unique sequence	Conserved sequence
HA 1	5′UUAUCAGCU	AGCAAUCACAUC
HA 2	5′AGAGUCCAUUC	AGCAAUCACAUC
PB1	5′ACAGCGCUCCU	AGCAAUCACAAU

TABLE 4. Results of HI tests with QRFV, LKCV, JAV, Thogoto virus, Dhori virus, and Araguari virus

Immune ascitic fluid	HI result[a] with antigen from:				
	QRFV	LKCV	Thogoto virus	Dhori virus	Araguari virus
QRFV	1,280	1,280	10	20	10
JAV[b]	80	20	0	0	0
LKCV	40	1,280	40	10	
Thogoto virus	0	0	≥1,280	80	10
Dhori virus	0	0	10	≥640	
Araguari virus	0	0	10	0	≥640

[a] Reciprocal of highest antiserum dilution against 4 U of antigen at pH 5.75.
[b] JAV did not produce an agglutinin at pH 5.75 to 7.0.

Serologic studies and sequencing of JAV and LKCV. Serologic analysis of JAV defined a close relationship between this virus and QRFV (11, 38). The close relationship between QRFV and JAV was confirmed by CF and HI testing (Tables 4 and 5). Although the HA from QRFV was most similar to the thogotoviruses, there was only minimal cross-reaction between these viruses and QRFV by CF and no relationship by HI testing. An additional unclassified virus, LKCV, is also antigenically related to QRFV and JAV (Tables 4 and 5). Electron microscopy of JAV-infected cells revealed pleomorphic virus-like particles with striking similarity to those seen in QRFV-infected cultures (data not shown). To assess whether a similar relationship exists between these three viruses at the sequence level, high-throughput sequencing of RNA extracted from JAV- or LKCV-infected Vero cells was performed. Two contigs that had detectable sequence similarity to known orthomyxoviruses and were closely related to the corresponding QRFV sequences were generated from JAV. One contig of 2,325 bp had 82% amino acid identity to QRFV PB1. The second contig was 1,229 bp, and shared 70% amino acid identity with QRFV HA. These sequences were confirmed by RT-PCR, cloning, and sequencing. The PB1 sequence contained a single ORF of 2,313 nt (770 amino acids). The HA sequence contained a single ORF of 1,092 nt (363 amino acids). The degree of sequence divergence seen between QRFV and JAV was similar to that seen between the two species in the genus *Thogotovirus*, Thogoto virus and Dhori virus. Phylogenetic

TABLE 5. Results of CF tests with QRFV, LKCV, JAV, Thogoto virus, Dhori virus, and Araguari virus

Antigen	CF result[a] with immune ascitic fluid from:					
	QRFV	JAV	LKCV	Thogoto virus	Dhori virus	Araguari virus
QRFV	256/32	0	8/8	0	0	0
JAV	0	32/≥8	0	0	0	0
LKCV	8/8	0	128/≥32	0	0	0
Thogoto virus	0	0	0	32/≥8	0	0
Dhori virus	0	0	0	0	64/≥4	0
Araguari virus	0	0	0	0	0	32/≥8

[a] Reciprocal of highest antiserum dilution/reciprocal of highest antigen dilution. 0 = <8/<8.

analysis demonstrated that JAV is most closely related to QRFV (Fig. 3A and B). In further support of this relationship, 5′ RACE of the JAV HA viral genome revealed the same conserved sequence motif at the 5′ end which had been observed in the QRFV HA: 5′-AGCAAUCACAAUCCUCUU UUUU. Partial sequencing of LKCV yielded a 715-bp fragment which shared 30 to 45% amino acid identity to regions of influenza B virus PB1, and 83% sequence identity to a region of QRFV PB1, consistent with its serologic relationship to QRFV and JAV and placing it as a likely third member of this viral serogroup.

DISCUSSION

The family *Orthomyxoviridae* had previously been comprised of five genera, *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Isavirus*, and *Thogotovirus*. Influenza viruses are important human pathogens, causing yearly outbreaks of respiratory infection and periodic severe epidemics resulting in thousands to millions of deaths annually (21). The genus *Isavirus* has only one member, which causes severe anemia in multiple fish strains and is a significant commercial problem. The thogotoviruses consist of three viruses: Thogoto virus, Dhori virus, and Araguari virus. Dhori virus has been reported to cause encephalitis and viral illness after the accidental infection of five laboratory workers (8). Because of the genetic ability of the orthomyxoviruses within a given genus to undergo reassortment of their genome segments, with the resultant emergence of new virus strains, these viruses pose a continual threat for epidemic outbreaks of disease in humans, poultry, and livestock.

Phylogenetic and sequence analysis of the complete predicted ORFs from both QRFV and JAV demonstrated a clear relationship with members of the family *Orthomyxoviridae*. Based on the observed phylogenetic distances, we propose that QRFV and JAV should be classified into a novel genus in this family, tentatively named *Quarjavirus*. This genus designation is further supported by the unique end-terminal sequence seen in the sequencing of both QRFV and JAV. Limited sequencing and serologic analysis support the addition of a third virus, LKCV, as a third member of this genus. The ultrastructural, morphological, and serologic studies presented in this paper further support the classification of these viruses as novel orthomyxoviruses.

The genomes of orthomyxoviruses consist of six to eight segments of negative single-strand RNA. In this study, we have generated sequence data demonstrating that QRFV and JAV are multisegmented RNA viruses. We have identified and completely sequenced five segments from QRFV, including four segments which we predict to encode proteins which are essential to the orthomyxovirus replication machinery: PA, PB1, PB2, and a glycoprotein sequence which is likely to function as the viral entry/fusion protein/HA. We have not definitively identified NP or M, nor have we identified any sequence which shares significant homology with the influenza virus neuraminidase or nonstructural segments. It is possible that the fifth segment encodes one of these functions, although no homology was detected. It is likely that additional segments exist but were not amplified under the conditions we used. One potential reason may be that the end-terminal sequence is not completely conserved among all segments. This has precedent, as the 3′-terminal sequence of Thogoto virus segment 6 is not conserved with the other segments, differing at three nucleotides (17).

The ends of orthomyxovirus segments are characterized by conserved sequences which show partial reverse complementarity (14). The conservation of these terminal sequences is thought to be important to the binding of the viral polymerase subunit, PB1, and activation of a unique process called cap snatching, which is enabled by the polymerase subunit, PB2 (6, 16, 33). Priming of viral mRNA synthesis in influenza viruses occurs by stealing capped fragments of 10 to 13 nt from the host (these are m^7GpppX^m-containing RNA fragments derived by cleavage of host cell RNA polymerase II transcripts) (21). In contrast, while Thogoto virus mRNA is capped, 5′ RACE analysis has determined that Thogoto virus mRNAs do not contain any significant heterogeneous sequence (35, 36). 5′ RACE of mRNA from QRFV identified 9 to 11 nucleotides which are heterogeneous among the different RACE products (Table 3). We hypothesize that this observed heterogeneity derives from host sequences captured in a cap-snatching process more similar to that described for influenza A than to that described for Thogoto virus.

While QRFV likely shares features of its mRNA synthesis process with the influenza viruses, it also resembles the thogotoviruses in other aspects, including its growth and tropism features. QRFV and Thogoto virus grow more slowly than the influenza viruses, with significant virus output at 24 h postinfection (7). QRFV and JAV, like Thogoto and Dhori viruses, are thought to be tick borne. Perhaps because of this difference in tropism, the HA sequence determined for QRFV shares greater sequence similarity with baculovirus glycoproteins and the HA of thogotoviruses, which are also more closely related to the baculovirus glycoproteins, than to the influenza virus HA proteins (Fig. 3B) (26). This might also explain why no neuraminidase sequence has been identified. Thus, QRFV shares features with both the influenza viruses and the thogotoviruses, further supporting its classification as a unique orthomyxovirus genus.

QRFV was initially isolated from two children with self-limited viral illness from the area of Quaranfil, Egypt (32). Serologic studies in the 1960s indicated that up to 8% of the local population had been exposed to QRFV (32). Aside from these studies, the prevalence of QRFV in the human population has not been assessed, nor has there been extensive effort made to determine the possible contributions of QRFV to human disease. The isolation of QRFV, JAV, and LKCV from ticks and birds in Africa, Central Asia, and the Pacific demonstrates that these viruses can be found over a wide geographic area, so a large fraction of the human population has potential exposure to this group of viruses. Classification and sequencing of these viruses are important first steps to developing assays to determine the extent of human infection caused by them and to understanding the basic virology and pathogenesis of these novel orthomyxoviruses.

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REFERENCES

1. **Al-Khalifa, M. S., F. M. Diab, and G. M. Khalil.** 2007. Man-threatening viruses isolated from ticks in Saudi Arabia. Saudi Med. J. **28:**1864–1867.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Austin, F. J.** 1978. Johnston Atoll virus (Quaranfil group) from Ornithodoros capensis (Ixodoidea: Argasidae) infesting a gannet colony in New Zealand. Am. J. Trop. Med. Hyg. **27:**1045–1048.
4. **Baskerville, A., and G. Lloyd.** 1976. The pathogenesis and pathology of experimental Quaranfil virus infection. Br. J. Exp. Pathol. **57:**152–156.
5. **Beaty, B. J., C. H. Calisher, and R. E. Shope.** 1989. Arboviruses, p. 797–855. *In* N. J. Schmidt and R. W. Emmons (ed.), Diagnostic procedures for viral, rickettsial, and chlamydial infections. American Public Health Association, Washington, DC.
6. **Biswas, S. K., and D. P. Nayak.** 1996. Influenza virus polymerase basic protein 1 interacts with influenza virus polymerase basic protein 2 at multiple sites. J. Virol. **70:**6716–6722.
7. **Booth, T. F., C. R. Davies, L. D. Jones, D. Staunton, and P. A. Nuttall.** 1989. Anatomical basis of Thogoto virus infection in BHK cell culture and in the ixodid tick vector, Rhipicephalus appendiculatus. J. Gen. Virol. **70:**1093–1104.
8. **Butenko, A. M., E. V. Leschinskaya, I. V. Semashko, M. A. Donets, L. I. Martiyanova, I. N. Martynenko, S. G. Rubin, and M. P. Chumakov.** 1987. Dhori virus, a causative agent of human disease: 5 cases of laboratory infection. Voprosy Virusologii **1987:**724–729.
9. **Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins, and J. D. Thompson.** 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. **31:**3497–3500.
10. **Clarke, D. H., and J. Casals.** 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg. **7:**561–573.
11. **Clifford, C. M., L. A. Thomas, L. E. Hughes, G. M. Kohls, and C. B. Philip.** 1968. Identification and comparison of two viruses isolated from ticks of the genus Ornithodoros. Am. J. Trop. Med. Hyg. **17:**881–885.
12. **Converse, J. D., and M. I. Moussa.** 1982. Quaranfil virus from Hyalomma dromedarii (Acari: Ixodoidea) collected in Kuwait, Iraq and Yemen. J. Med. Entomol. **19:**209–210.
13. **Da Silva, E. V., A. P. Da Rosa, M. R. Nunes, J. A. Diniz, R. B. Tesh, A. C. Cruz, C. M. Vieira, and P. F. Vasconcelos.** 2005. Araguari virus, a new member of the family Orthomyxoviridae: serologic, ultrastructural, and molecular characterization. Am. J. Trop. Med. Hyg. **73:**1050–1058.
14. **Desselberger, U., V. R. Racaniello, J. J. Zazra, and P. Palese.** 1980. The 3′ and 5′-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. Gene. **8:**315–328.
15. **Guindon, S., and O. Gascuel.** 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52:**696–704.
16. **Hagen, M., L. Tiley, T. D. Chung, and M. Krystal.** 1995. The role of template-primer interactions in cleavage and initiation by the influenza virus polymerase. J. Gen. Virol. **76:**603–611.
17. **Hagmaier, K., S. Jennings, J. Buse, F. Weber, and G. Kochs.** 2003. Novel gene product of Thogoto virus segment 6 codes for an interferon antagonist. J. Virol. **77:**2747–2752.
18. **Hulo, N., A. Bairoch, V. Bulliard, L. Cerutti, B. A. Cuche, E. de Castro, C. Lachaize, P. S. Langendijk-Genevaux, and C. J. Sigrist.** 2008. The 20 years of PROSITE. Nucleic Acids Res. **36:**D245–D249.
19. **Hulo, N., A. Bairoch, V. Bulliard, L. Cerutti, E. de Castro, P. S. Langendijk-Genevaux, M. Pagni, and C. J. Sigrist.** 2006. The PROSITE database. Nucleic. Acids Res. **34:**D227–D230.
20. **Kemp, G. E., V. H. Lee, and D. L. Moore.** 1975. Isolation of Nyamanini and Quaranfil viruses from Argas (Persicargas) arboreus ticks in Nigeria. J. Med. Entomol. **12:**535–537.
21. **Lamb, R. A., and R. M. Krug.** 2001. Orthomyxoviridae, p. 1487–1532. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology. Lippincott Williams & Wilkins, Philadelphia, PA.
22. **Lin, D. A., S. Roychoudhury, P. Palese, W. C. Clay, and F. J. Fuller.** 1991. Evolutionary relatedness of the predicted gene-product of RNA segment-2 of the tick-borne Dhori virus and the Pb1 polymerase gene of influenza-viruses. Virology **182:**1–7.
23. **Marchler-Bauer, A., J. B. Anderson, P. F. Cherukuri, C. Weese-Scott, L. Y. Geer, M. Gwadz, S. He, D. I. Hurwitz, J. D. Jackson, Z. Ke, C. J. Lanczycki, C. A. Liebert, C. Liu, F. Lu, G. H. Marchler, M. Mullokandov, B. A. Shoemaker, V. Simonyan, J. S. Song, P. A. Thiessen, R. A. Yamashita, J. J. Yin, D. Zhang, and S. H. Bryant.** 2005. CDD: a Conserved Domain Database for protein classification. Nucleic Acids Res. **33:**D192–D196.
24. **Marchler-Bauer, A., J. B. Anderson, F. Chitsaz, M. K. Derbyshire, C. Weese-Scott, J. H. Fong, L. Y. Geer, R. C. Geer, N. R. Gonzales, M. Gwadz, S. He, D. I. Hurwitz, J. D. Jackson, Z. Ke, C. J. Lanczycki, C. A. Liebert, C. Liu, F. Lu, S. Lu, G. H. Marchler, M. Mullokandov, J. S. Song, A. Tasneem, N. Thanki, R. A. Yamashita, D. Zhang, N. Zhang, and S. H. Bryant.** 2009. CDD: specific functional annotation with the Conserved Domain Database. Nucleic Acids Res. **37:**D205–D210.
25. **McBeath, A. J., B. Collet, R. Paley, S. Duraffour, V. Aspehaug, E. Biering, C. J. Secombes, and M. Snow.** 2006. Identification of an interferon antagonist protein encoded by segment 7 of infectious salmon anaemia virus. Virus. Res. **115:**176–184.
26. **Morse, M. A., A. C. Marriott, and P. A. Nuttall.** 1992. The glycoprotein of Thogoto virus (a tick-borne orthomyxo-like virus) is related to the baculovirus glycoprotein GP64. Virology **186:**640–646.
27. **Pollock, J. L., R. M. Presti, S. Paetzold, and H. W. Virgin.** 1997. Latent murine cytomegalovirus infection in macrophages. Virology **227:**168–179.
28. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27:**493–497.
29. **Sang, R., C. Onyango, J. Gachoya, E. Mabinda, S. Konongoi, V. Ofula, L. Dunster, F. Okoth, R. Coldren, R. Tesh, A. T. da Rosa, S. Finkbeiner, D. Wang, M. Crabtree, and B. Miller.** 2006. Tickborne arbovirus surveillance in market livestock, Nairobi, Kenya. Emerg. Infect. Dis. **12:**1074–1080.
30. **Sureau, P., and J. M. Klein.** 1980. Arboviruses in Iran. Med. Trop. (Mars.) **40:**549–554.
31. **Swofford, D. L.** 2000. PAUP*. Phylogenetic analysis using parsimony (*and other methods), v. 4.0. Sinauer Associates, Sunderland, MA.
32. **Taylor, R. M., H. S. Hurlbut, T. H. Work, J. R. Kingston, and H. Hoogstraal.** 1966. Arboviruses isolated from Argas ticks in Egypt: Quaranfil, Chenuda, and Nyamanini. Am. J. Trop. Med. Hyg. **15:**76–86.
33. **Tiley, L. S., M. Hagen, J. T. Matthews, and M. Krystal.** 1994. Sequence-specific binding of the influenza virus RNA polymerase to sequences located at the 5′ ends of the viral RNAs. J. Virol. **68:**5108–5116.
34. **Wang, D., L. Coscoy, M. Zylberberg, P. C. Avila, H. A. Boushey, D. Ganem, and J. L. DeRisi.** 2002. Microarray-based detection and genotyping of viral pathogens. Proc. Natl. Acad. Sci. USA **99:**15687–15692.
35. **Weber, F., O. Haller, and G. Kochs.** 1996. Nucleoprotein viral RNA and mRNA of Thogoto virus: a novel "cap-stealing" mechanism in tick-borne orthomyxoviruses? J. Virol. **70:**8361–8367.
36. **Weber, F., O. Haller, and G. Kochs.** 1997. Conserved vRNA end sequences of Thogoto-orthomyxovirus suggest a new panhandle structure. Arch. Virol. **142:**1029–1033.
37. **Xu, F., D. Liu, M. R. Nunes, A. P. Da Rosa, R. B. Tesh, and S. Y. Xiao.** 2007. Antigenic and genetic relationships among Rift Valley fever virus and other selected members of the genus Phlebovirus (Bunyaviridae). Am. J. Trop. Med. Hyg. **76:**1194–1200.
38. **Zeller, H. G., N. Karabatsos, C. H. Calisher, J. P. Digoutte, F. A. Murphy, and R. E. Shope.** 1989. Electron microscopy and antigenic studies of uncharacterized viruses. I. Evidence suggesting the placement of viruses in families Arenaviridae, Paramyxoviridae, or Poxviridae. Arch. Virol. **108:**191–209.